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Soluble flagellin, FliC, induces an Ag-specific Th2 response, yet promotes T-bet-regulated Th1 clearance of *Salmonella typhimurium* infection

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Clearance of disseminated *Salmonella* infection requires bacterial-specific Th1 cells and IFN- γ production, and Th1-promoting vaccines are likely to help control these infections. Consequently, vaccine design has focused on developing Th1-polarizing adjuvants or Ag that naturally induce Th1 responses. In this study, we show that, in mice, immunization with soluble, recombinant FliC protein flagellin (sFliC) induces Th2 responses as evidenced by Ag-specific GATA-3, IL-4 mRNA, and protein induction in CD62L^{lo} CD4⁺ T cells without associated IFN- γ production. Despite these Th2 features, sFliC immunization can enhance the development of protective Th1 immunity during subsequent *Salmonella* infection in an Ab-independent, T-cell-dependent manner. *Salmonella* infection in sFliC-immunized mice resulted in augmented Th1 responses, with greater bacterial clearance and increased numbers of IFN- γ -producing CD4⁺ T cells, despite the early induction of Th2 features to sFliC. The augmented Th1 immunity after sFliC immunization was regulated by T-bet although T-bet is dispensable for primary responses to sFliC. These findings show that there can be flexibility in T-cell responses to some subunit vaccines. These vaccines may induce Th2-type immunity during primary immunization yet promote Th1-dependent responses during later infection. This suggests that designing Th1-inducing subunit vaccines may not always be necessary since this can occur naturally during subsequent infection.

Keywords: Flagellin · *Salmonella* · T-bet · T cells · Vaccine

Introduction

Vaccination has been a major factor in improving life-expectancy through inducing effective Ab and/or T-cell-mediated immunity.

Licensed vaccines are usually one of the three types: inactivated whole pathogen; live, attenuated organism, or subunits of the pathogen. All three can be given alone or in the presence of adjuvant. There are no licensed vaccines for use in humans against nontyphoidal *Salmonella* infections such as those caused by *Salmonella enterica* serovar Typhimurium (STm), despite it being a

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leading cause of death in infants in regions such as sub-Saharan Africa [1]. On the contrary, there are three distinct vaccines that offer good but limited protection against typhoid, caused by *Salmonella enterica* serovar Typhi (ST) [2–4]. While killed vaccine is rarely used because of its toxicity, live attenuated Ty21a vaccine and purified capsular polysaccharide Vi Ag, confer protection against typhoid in adults. Importantly, they indicate that multiple Ag and mechanisms can be used to protect against *Salmonella* infections. Thus, ST Ty21a induces potent systemic and mucosal T- and B-cell responses [5], whereas purified Vi Ag induces T-independent serum Ab, but poor mucosal responses [4]. Significantly, despite lacking Vi Ag and LPS O-chain Ty21a can protect against typhoid and thus other Ag may be useful in subunit vaccines [6].

Salmonella infections can be modelled effectively in the mouse [7, 8] but to study adaptive immunity to *Salmonella* in susceptible mouse strains (e.g. C57BL/6 or BALB/c), attenuated bacterial strains are commonly used. Clearance of primary infections requires IFN- γ and T-bet-regulated differentiation of CD4⁺ T cells to Th1 [9–14]. In contrast, B cells and CD8⁺ T cells are not required to clear primary infections, although Ab can both moderate bacteremia and help protect against reinfection [9, 15–18]. Mouse models allow candidate vaccines to be tested for their potential to protect against *Salmonella* infections and enables analysis of the mechanisms by which they protect. Thus, we recently showed that Ab to OmpD, absent in ST, can inhibit infection [19, 20] in a T-independent manner.

A common feature of Vi Ag and OmpD is that they are cell surface localized. Another surface-exposed Ag is flagellin. In STm, there are two flagellin genes, *fliC* and *fliB*, and cells may express one or other of these genes but not both concurrently. Flagellin interacts with the immune system directly through at least two pathways [21–23]: through TLR5 or via recognition by NOD-like receptor NLRC4. The impact of flagellin on the innate immune system is profound and includes the induction of cytokines from multiple cell types, DC maturation, and adaptive responses to itself and coadministered Ag [24–31].

The capacity of bacterial flagellin to be both a target of the immune response and an adjuvant are well reported [25, 26, 32–35]. More surprising is that, although it is a TLR ligand, it provokes T- and B-cell responses with a strong Th2 bias to itself and coadministered Ag [25–27, 29]. Nevertheless, isotype switching to flagellin when surface attached to STm is Th1 reflecting [25]. Thus, the direction of the in vivo response to flagellin is influenced by the context that the Ag is encountered by the host immune system.

In this study, we have assessed how the immune response to soluble, recombinant FliC protein flagellin (sFliC) affects subsequent STm infection. Primary immunization with sFliC induces Th2 responses. Nevertheless, when sFliC-immunized mice are challenged they show enhanced T-cell-dependent, Ab-independent resistance to STm infection. This is because prior sFliC immunization augments the numbers of IFN- γ -producing Th1 cells during subsequent STm infection via a T-bet-regulated mechanism. The broad significance of this study is that although sFliC drives a clear Th2 response it still primes for enhanced

T-cell-mediated protection during subsequent STm infection. This provides an example of a beneficial flexibility in the direction of T-cell-mediated help induced by a subunit vaccine.

Results

Soluble flagellin induces Th2 responses during primary immunization

To test whether the CD4⁺ T-cell response to soluble flagellin is Th2, we examined responses in WT and transgenic, flagellin-specific SM1 CD4⁺ T cells [36]. First, CFSE-labeled, SM1 CD4⁺ T cells were adoptively transferred into WT mice to compare the kinetics of the response post-immunization with sFliC or STm. Almost, all splenic SM1 CD4⁺ T cells had undergone four or more rounds of division by 48 h in chimeras immunized with sFliC, whereas 80–90% of the transferred cells from those infected with STm had completed between one and four cell cycles. The SM1 CD4⁺ T cells in both groups of immunized chimeras showed an activated profile as assessed by induced CD69 expression and loss of CD62L (Fig. 1A).

Th features induced by sFliC or STm were then assessed 4–7 days after immunization, depending on the experiment. Real-time RT-PCR was used to assess gene expression in total CD4⁺ T cells from nonimmunized (NI) WT mice or after sFliC or STm in WT or SM1 CD4⁺ T cells sorted into CD62L^{lo} (effector) or CD62L^{hi} (noneffector) subsets. These experiments show that CD62L^{lo} effector T cells from sFliC-immunized mice had upregulated GATA-3 and IL-4 mRNA compared with STm-infected mice. On the contrary, IL-4 mRNA was largely undetectable in the absence of sFliC immunization (Fig. 1B). IFN- γ and T-bet mRNA levels were consistently higher after STm infection than in NI mice or sFliC-immunized mice. While some expression of IFN- γ and T-bet mRNA was seen in CD62L^{lo} effector T cells after sFliC, levels were approximately 10–100-fold lower than in CD62L^{lo} effector T cells from STm-immunized mice. IL-17 mRNA expression was not detected in any population under any condition (data not shown). To confirm that IL-4 mRNA induction reflected IL-4 protein production, we performed ELISPOT analysis on sFliC-restimulated endogenous or SM1 T cells from NI mice or STm- or sFliC-immunized mice (Fig. 1C). This showed that in both endogenous and transgenic T cells there was a marked increase in IL-4 spot-forming cells (SFC) after sFliC, but not STm. This indicates that sFliC immunization induces IL-4 mRNA and protein-producing cells.

Next, we examined IFN- γ production 7 days after immunization with sFliC or STm by intracellular FACS staining after restimulation with anti-CD3 or sFliC. We found that sFliC, unlike STm, fails to induce IFN- γ production in WT CD4⁺ T cells. As seen previously [37], IFN- γ production after STm was found in CD62L^{lo} WT T cells (Fig. 2A). Similar results were observed in SM1 T cells from chimeras, with IFN- γ induced after STm but to much lower levels after sFliC (Fig. 2A). Restimulation with sFliC rather than anti-CD3 Ab showed similar results, except that in WT cells IFN- γ levels were half those after anti-CD3 stimulation,

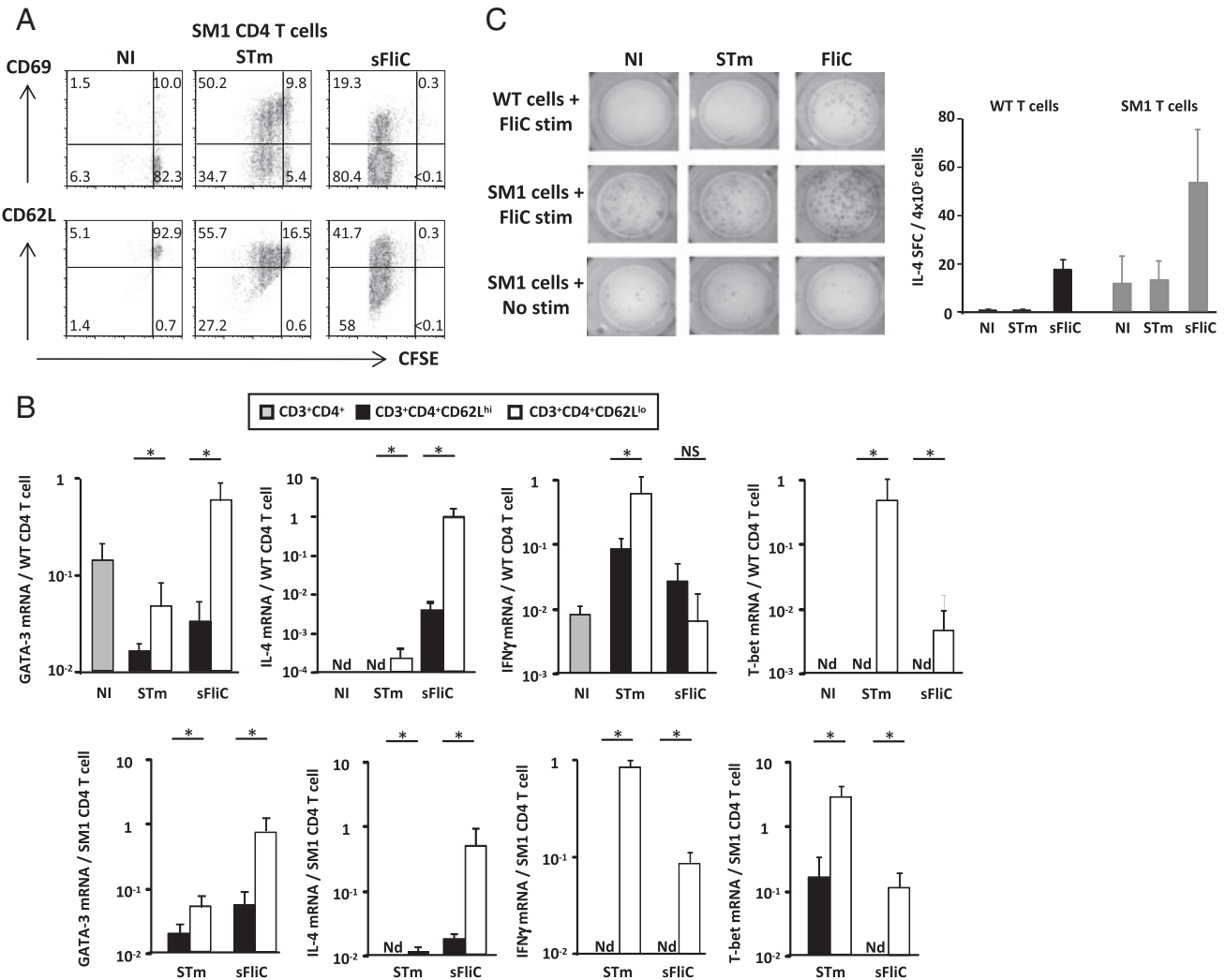


Figure 1. Soluble flagellin induces Th2 responses. (A) Splenocytes (10^7) from transgenic flagellin-specific SM1 mice were CFSE-labeled before adoptive transfer into WT mice. Chimeras were either NI or after 24 h received i.p. 5×10^5 STm or $20 \mu\text{g}$ sFliC for 48 h before splenic SM1 CD4⁺ T-cell division and activation was assessed by CFSE dilution and CD69 or CD62L expression. FACS plots are representative of three experiments. (B) Graphs show relative mRNA expression per cell (mean ± SD) for GATA-3, IL-4, IFN- γ , and T-bet by real-time RT-PCR on FACS-sorted NI CD4⁺ T cells (gray bars), CD62L^{hi} CD4⁺ T cells (black bars), or CD62L^{lo} CD4⁺ T cells (white bars) from WT mice (sorted on CD3, CD4, and CD62L expression; top panels) or SM1/Rag1-deficient chimeras (gated on CD3, CD4, and CD62L expression; all T cells are SM1 cells; bottom panels) that received 5×10^5 STm or $20 \mu\text{g}$ sFliC for 4 days i.p. (C) Splenocytes from NI WT mice or Rag1 SM1 chimeras, or WT mice or Rag1 SM1 chimeras immunized with STm or sFliC for 5 days were isolated and restimulated for 48 h before the numbers of IL-4 SFC were enumerated by ELISPOT. Graph shows mean ± SD. Nd, not detected; $p \leq 0.05$ by the Mann-Whitney test. In all panels, data are representative of ≥ 2 experiments.

reflecting previous reports [34, 38]. ELISPOT experiments for IFN- γ secretion confirmed that intracellular IFN- γ protein production reflected protein secretion (data not shown). After sFliC <1% of splenic CD4⁺ T cells responding to sFliC produced IL-17 or TNF- α protein and there were only small changes in T-cell proportions expressing FoxP3 or BCL-6 (data not shown). Next, we assessed whether the low proportion of IFN- γ -producing T cells induced by sFliC reflected levels induced in WT and OVA-specific OTII CD4⁺ T cells after immunization with the model Th2 Ag alum-precipitated OVA (Fig. 2B). Similar levels of IFN- γ were induced in WT or OTII cells from WT-OTII chimeras by alum-precipitated OVA as by sFliC, showing that the levels of IFN- γ induced by sFliC are no greater than those induced to other Th2 Ags. To assess whether the poor IFN- γ responses induced to sFliC

simply reflected the use of monomeric sFliC, WT mice were immunized with polymeric flagellar filaments isolated from the surface of STm, which failed to promote IFN- γ production (Fig. 2C). The transcription factor T-bet is required for Th1 development [39]. Assessment of its expression showed that STm but not sFliC induced its induction in endogenous CD4⁺ T cells 7 days after immunization (Fig. 2D). It was possible that sFliC selectively induced IFN- γ in sites other than the spleen, such as the MLN. To assess this, we performed an ELISPOT assay on WT T cells in the MLN 5 days after immunization with sFliC (Fig. 2E). This showed that IL-4, but not IFN- γ -secreting, cells could be readily detected after sFliC immunization. Finally, IFN- γ -producing cells after sFliC immunization did not appear later in the response as they remained at background levels 35

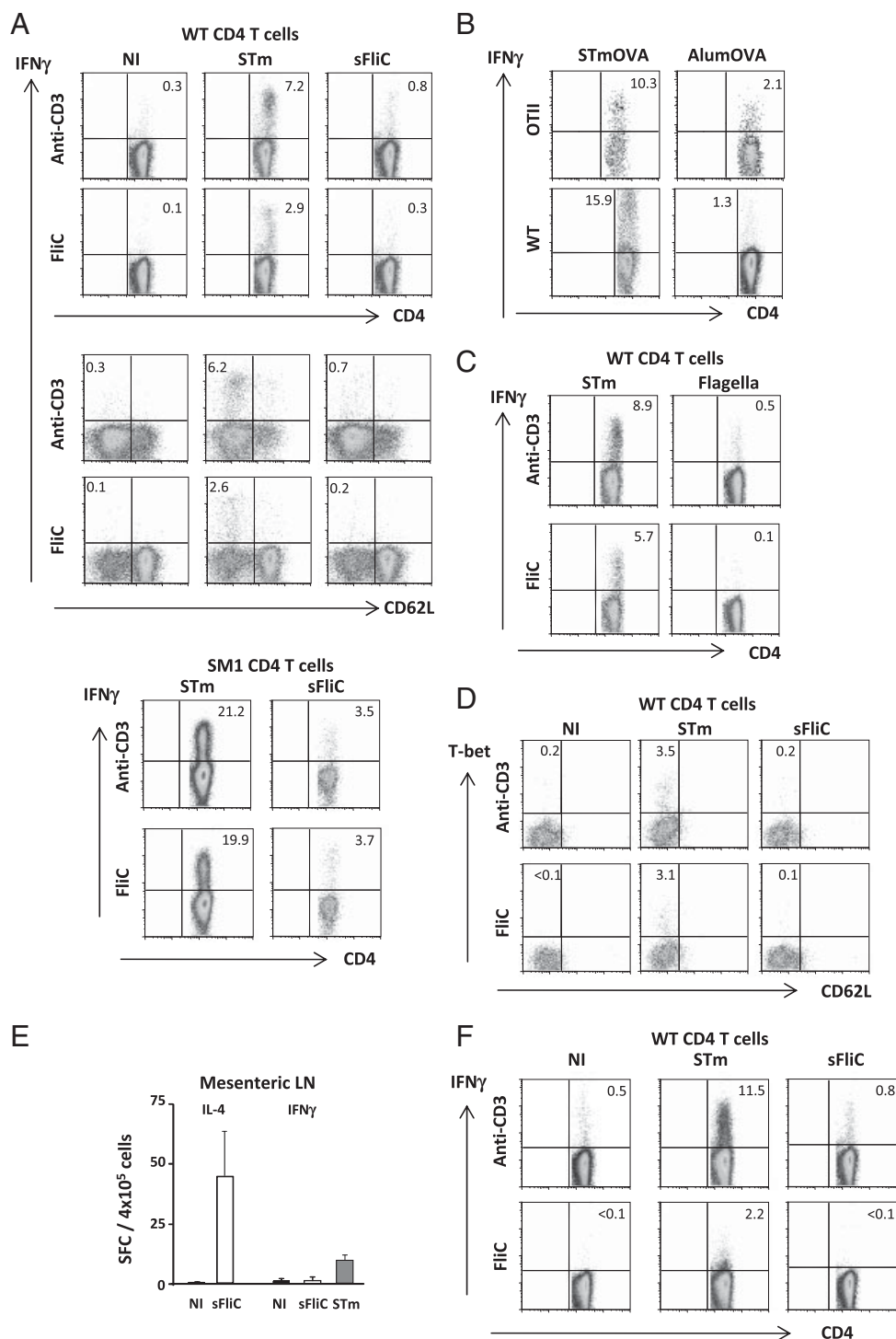


Figure 2. Immunization with soluble flagellin fails to induce IFN- γ or T-bet. (A) Representative FACS plots of intracellular IFN- γ production after restimulation with anti-CD3 Ab or sFliC in total WT CD4⁺ T cells (top two rows) or WT T cells subdivided by CD62L expression (middle two rows) or SM1 CD4⁺ T cells from SM1/WT chimeras (gated on $\nu\beta 2$; bottom two rows) given 5×10^5 STm or $20 \mu\text{g}$ sFliC i.p. for 7 days. (B) Representative FACS plots of intracellular IFN- γ production in OTII cells from chimeras (gated on CD45.1) or WT CD4⁺ T cells given 5×10^5 STmOVA or $50 \mu\text{g}$ alum-precipitated OVA i.p. for 7 days. (C) Representative FACS plots of intracellular IFN- γ production after restimulation of WT CD4⁺ T cells from mice given 5×10^5 STm or $20 \mu\text{g}$ surface-purified flagella i.p. for 7 days with anti-CD3 Ab or sFliC. (D) Representative FACS plots of T-bet expression in WT CD62L^{hi} and CD62L^{lo} CD4⁺ T cells from NI mice or mice given 5×10^5 STm or $20 \mu\text{g}$ sFliC i.p. for 7 days after restimulation with anti-CD3 Ab or sFliC. (E) MLN from NI WT mice, or WT mice immunized with STm or sFliC for 5 days were isolated and restimulated with sFliC for 48 h before IL-4 or IFN- γ SFC were enumerated by ELISPOT. The graph shows mean \pm SD from one of the two independent experiments; four mice per group. (F) Representative FACS of intracellular IFN- γ production in WT total CD4⁺ T cells given 5×10^5 STm or $20 \mu\text{g}$ sFliC i.p. for 35 days. In all cases, FACS plots are representative of ≥ 2 independent experiments with four mice per group.

days after immunization (Fig. 2F). The data shown in Figs. 1 and 2 indicate that soluble flagellin induces Th2 responses *in vivo*, with GATA-3 upregulation and pronounced IL-4 mRNA production, but poor IFN- γ and T-bet induction.

Immunization with soluble flagellin promotes clearance of STm at discrete stages of infection

Since flagellin induces Th2 immune responses (Fig. 1), we assessed whether immunization with sFliC could restrict subsequent systemic STm infection. To do this, we immunized WT mice with 20 μ g sFliC for 35 days before infecting *i.p.* with 5×10^6 STm and assessed splenic bacterial numbers 5, 18, and 35 days later. This showed that while sFliC conferred no benefit in controlling splenic STm infection on day 5 after infection there was an approximate 90% reduction in bacterial numbers by day 18 after infection and significant, but somewhat smaller, differences in bacterial burdens on day 35 after infection (Fig. 3). To examine whether systemic sFliC immunization promoted mucosal immunity, mice were *i.p.* immunized with 20 μ g sFliC for 35 days before oral challenge with 10^9 STm and bacterial colonization of the MLN and spleen assessed 2 days later. This showed that there was a small trend toward lower colonization of these tissues after sFliC immunization but this was not significant, despite groups containing at least seven mice (Fig. 3). Therefore, despite inducing Th2 responses, immunization with sFliC accelerates bacterial clearance after the first week of subsequent infection with STm.

Ab to sFliC fails to protect against STm infection

We have recently shown that Ab to heat-killed or subunit vaccines against STm is effective by day 5 after infection [19]. The similar bacterial numbers in NI and sFliC-immunized mice on day 5 after

infection suggest that Ab to sFliC does not inhibit bacterial colonization. We used a number of approaches to test this.

First, we confirmed [25] that sFliC or surface-isolated flagella immunization resulted in sustained IgG1 and IgG2a responses (Fig. 4A). Ab to sFliC was then tested to see if it could impair the motility of STm through agar. C-inactivated serum from an individual NI or sFliC-immunized mouse was added to an agar plate before bacteria were added and bacterial motility measured. Bacteria had impaired motility through agar that contained sFliC-specific serum relative to those containing sera from NI mice (Fig. 4B). Next, bacterial numbers were assessed in WT and B-cell-deficient mice primed with 20 μ g sFliC 35 days before infection with 5×10^6 STm (Fig. 4C). Five days later, WT and B-cell-deficient mice had similar levels of bacteria irrespective of whether they had been immunized with sFliC. We have previously shown [18, 19] that porins or STm induce Ab that can markedly reduce the number of STm that colonize the spleen. STm were incubated with C-inactivated anti-sFliC Ab, or anti-porin Ab, or anti-STm Ab immediately prior to *i.p.* infection into naïve mice (Fig. 4D). Although opsonization with anti-total STm or porin Ab markedly decreased bacterial colonization [18, 19], opsonization with anti-sFliC Ab did not. Finally, we examined whether the ability of STm to phase switch their flagellin expression accounts for this lack of benefit from sFliC immunization. To test this, WT mice were immunized with 20 μ g sFliC for 35 days and infected for 5 days with 5×10^5 STm or STm that express only FliC or FljB (Fig. 4E). FljB-locked STm bacterial numbers were not reduced after sFliC immunization but FliC-locked STm numbers were approximately tenfold lower. To exclude the possibility that FliC-locked bacteria were intrinsically more susceptible to killing by innate mechanisms, we infected T- and B-cell-deficient Rag1-deficient mice. This shows that all strains colonized equally well (Fig. 4E). Therefore, Ab to sFliC induced after immunization can restrict bacterial motility but not systemic bacterial colonization, partly through a capacity of STm to phase switch their flagella.

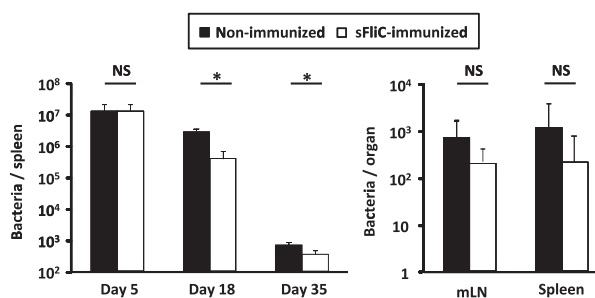


Figure 3. Flagellin immunization promotes clearance of STm after the first week of infection. Left panel, WT mice, NI or immunized *i.p.* with 20 μ g sFliC for 35 days were infected *i.p.* with 5×10^6 STm for 5, 18, or 35 days and splenic bacterial numbers enumerated. Right panel, WT mice, NI, or immunized *i.p.* with 20 μ g sFliC for 35 days were infected orally with 10^9 STm for 2 days and MLN and splenic bacterial numbers enumerated. * $p \leq 0.05$ by the Mann-Whitney test. Graphs show mean bacterial numbers \pm SD. Data are representative of ≥ 2 experiments at each time point with ≥ 4 mice in each group.

Enhanced bacterial clearance after sFliC correlates with increased IFN- γ -producing CD4⁺ T cell numbers

Since Ab to sFliC did not moderate infection, we next assessed whether the protection afforded by sFliC immunization on day 18 post-infection (Fig. 3 and Fig. 5A) was T-cell mediated. First, it was confirmed that T cells are not important for controlling STm infection in the first week of infection, but are necessary subsequently, by infecting WT and T-cell-deficient mice with 10^5 STm for 5 and 18 days (Fig. 5A). As expected [9, 19, 40, 41], on day 5 after infection both groups had similar splenic bacterial burdens, whereas at day 18 WT mice had significantly fewer bacteria than T-cell-deficient mice. It is not likely that the benefits of sFliC immunization were due to direct effects on the innate immune system since bacterial numbers in NI and sFliC immunized Rag1-deficient mice were similar on days 5 and day 18 after STm (Fig. 5B). Furthermore, infection of WT and T-cell-

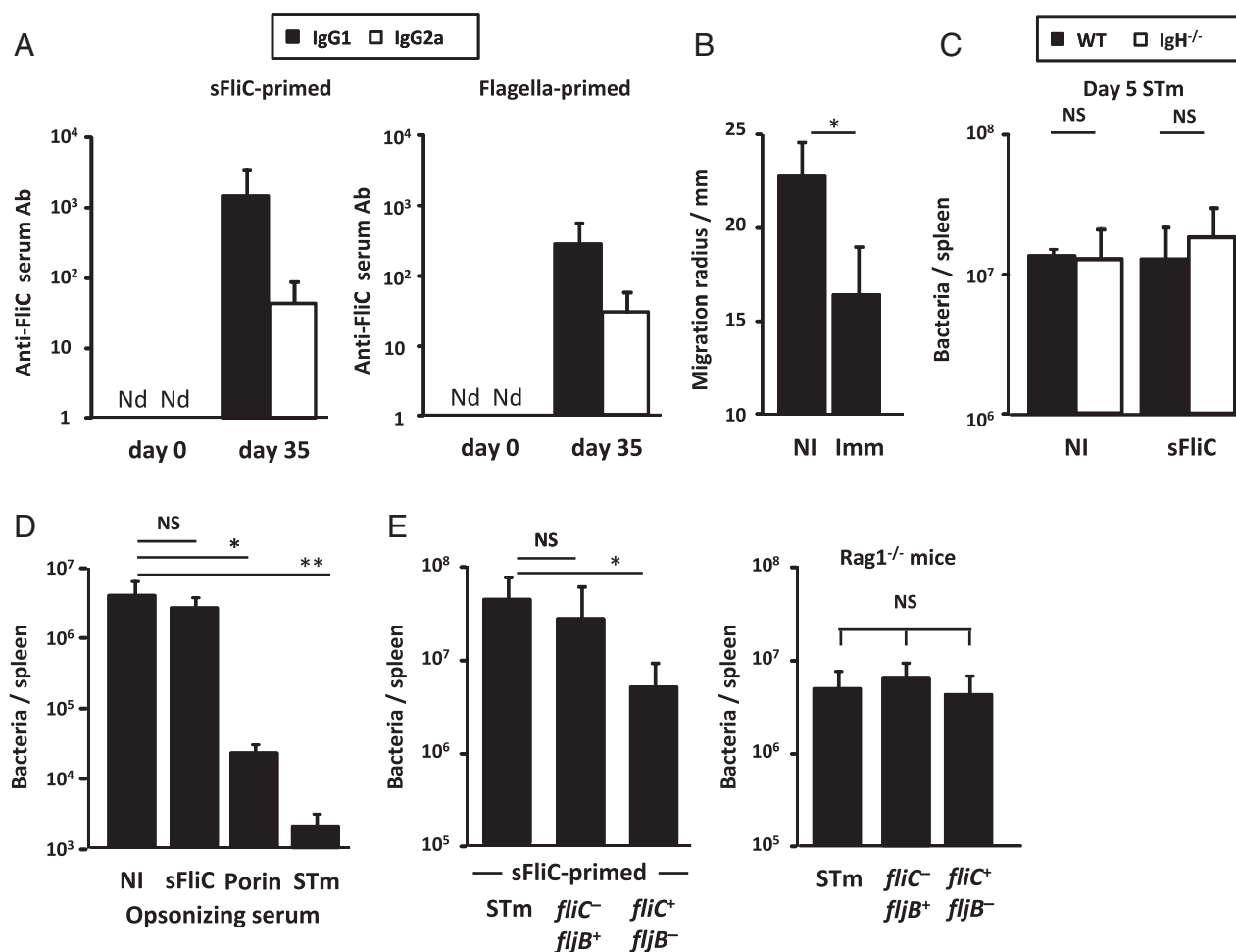


Figure 4. Flagellin induces potent Ab responses which fail to impair STm infection. (A) Serum FliC-specific IgG1 and IgG2a Ab titers induced 35 days after 20 μ g sFliC (left graph) or 20 μ g surface-purified flagellin (right graph) i.p. immunization. (B) Radius of bacterial swimzones after swimming through 0.3% agar containing C-inactivated sera from naïve mice (NI) or mice immunized twice with sFliC (four sera/group). (C) Splenic bacterial numbers in NI or sFliC-immunized (20 μ g for 35 days) WT or B-cell-deficient (IgH^{-/-}) mice infected for 5 days i.p. with 5 \times 10⁶ STm. (D) Splenic bacterial counts after i.p. infection for 5 days with 5 \times 10⁵ STm opsonized with C-inactivated NI sera, or anti-sFliC sera (14 days post-boosting), or anti-porin sera, or anti-STm sera. (E) Left graph: Splenic bacterial numbers in WT mice immunized for 35 days with 20 μ g sFliC before infection with 5 \times 10⁶ STm or *fliC*⁻*fljB*⁺ STm (only express *fljB*) or *fliC*⁺*fljB*⁻ STm (only express *fliC*). Right graph: Splenic bacterial numbers from Rag1-deficient mice infected i.p. with 5 \times 10⁵ of each strain for 7 days. Graphs show mean \pm SD; groups contain \geq 4 animals, sera, or bacterial cultures. Nd, not detected. * $p \leq 0.05$ by the Mann-Whitney test. Experiments are representative of ≥ 2 repeats in each case.

deficient mice with or without sFliC immunization for 35 days showed that T cells were important for the additional control of infection afforded by sFliC immunization at day 18 post-infection (Fig. 5B). In contrast, when these experiments were performed only to day 5 after infection bacterial burdens were similar in WT and T-cell-deficient mice independent of sFliC immunization (data not shown). Since clearance of STm requires IFN- γ , we assessed how previous sFliC immunization altered IFN- γ production in CD4⁺ T cells during subsequent infection. At day 5 after infection, proportions and numbers of IFN- γ producing CD4⁺ T cells were lower in sFliC-immunized mice (Fig. 5C). Nevertheless, when responses were assessed after 18 days of infection, the sFliC-immunized group had a higher proportion and number of IFN- γ ⁺ CD4⁺ T cells, with IFN- γ only detectable in CD62L^{lo} CD4⁺ T cells (Fig. 5C). By day 35, when infection has nearly resolved in both groups, the numbers and proportions of IFN- γ ⁺

CD4⁺ T cells in both groups were similar. These results were unlikely to be influenced by IL-4 since ELISPOT failed to identify the differences in immunized and NI groups on day 5 after infection and IL-4⁺ SFC were largely undetectable at day 18 after infection (Fig. 5D). Thus, under these conditions immunization with the Th2 Ag sFliC can promote Th1 responses.

T-bet is essential for enhanced bacterial clearance and IFN- γ production after sFliC immunization

Since sFliC enhanced IFN- γ responses to STm, we wished to assess how this was mediated. While antibody to sFliC did not help control infection at day 5 of infection, it remained possible that B cells and antibody contributed by day 18 when the benefit of sFliC immunization is apparent. We immunized WT and B-cell-

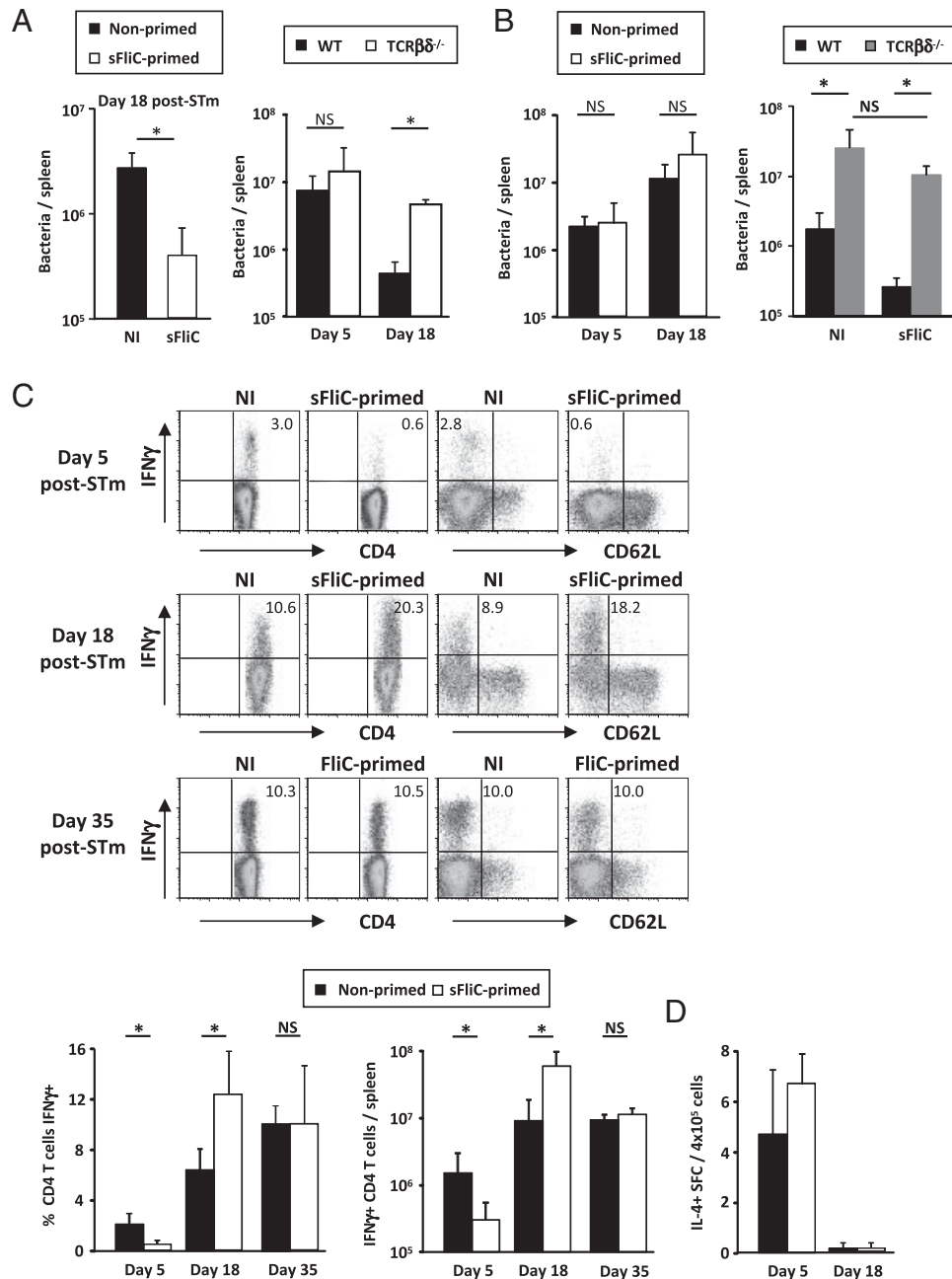


Figure 5. Enhanced bacterial clearance after sFliC immunization correlates with augmented numbers of IFN- γ producing CD4⁺ T cells. (A) Left graph: splenic bacterial numbers in naïve (NI) or sFliC-immunized mice infected for 35 days. Right graph: Splenic bacterial counts from WT and T-cell-deficient (TCR $\beta\delta^{-/-}$) mice infected i.p. with 5×10^5 STm for 5 and 18 days. (B) Left graph: Splenic bacterial counts in NI or sFliC-immunized (20 μ g; i.p. for 7 days) Rag1-deficient mice infected i.p. with 5×10^5 STm for 7 or 18 days. Right graph: Splenic bacterial numbers in NI or sFliC-immunized (20 μ g for 35 days) WT or T-cell-deficient mice infected for 18 days i.p. with 5×10^5 STm. (C) Representative FACS plots showing intracellular IFN- γ production, after anti-CD3 stimulation with anti-CD28, in total splenic CD4⁺ T cells or subdivided by CD62L expression in NI or sFliC-immunized (20 μ g for 35 days) WT mice infected i.p. with 5×10^5 STm for either 5, 18, or 35 days. Proportion (left graph) and total numbers (right graph) of IFN- γ -producing splenic CD4⁺ T cells. (D) Splenocytes from NI or sFliC-immunized WT mice infected with STm for 5 or 18 days were isolated and restimulated for 48 h before the numbers of IL-4 SFC were enumerated by ELISPOT. Graphs show mean and one SD. Groups contained four mice and experiments are representative of ≥ 2 repeats. * $p \leq 0.05$ by the Mann-Whitney test.

deficient mice for 35 days before infection with 5×10^5 STm and examined splenic bacterial numbers and levels of IFN- γ production 18 days later (Fig. 6A). This showed that the absence of B cells and antibody did not influence bacterial clearance or IFN- γ responses by CD4⁺ T cells, irrespective of whether mice had been

immunized with sFliC. These experiments were repeated using IL-4R α -deficient mice and showed that signaling through IL-4R α was dispensable for the sFliC-mediated control of infection or to IFN- γ production (data not shown). Next, we assessed whether the beneficial effects of sFliC immunization were regulated by the

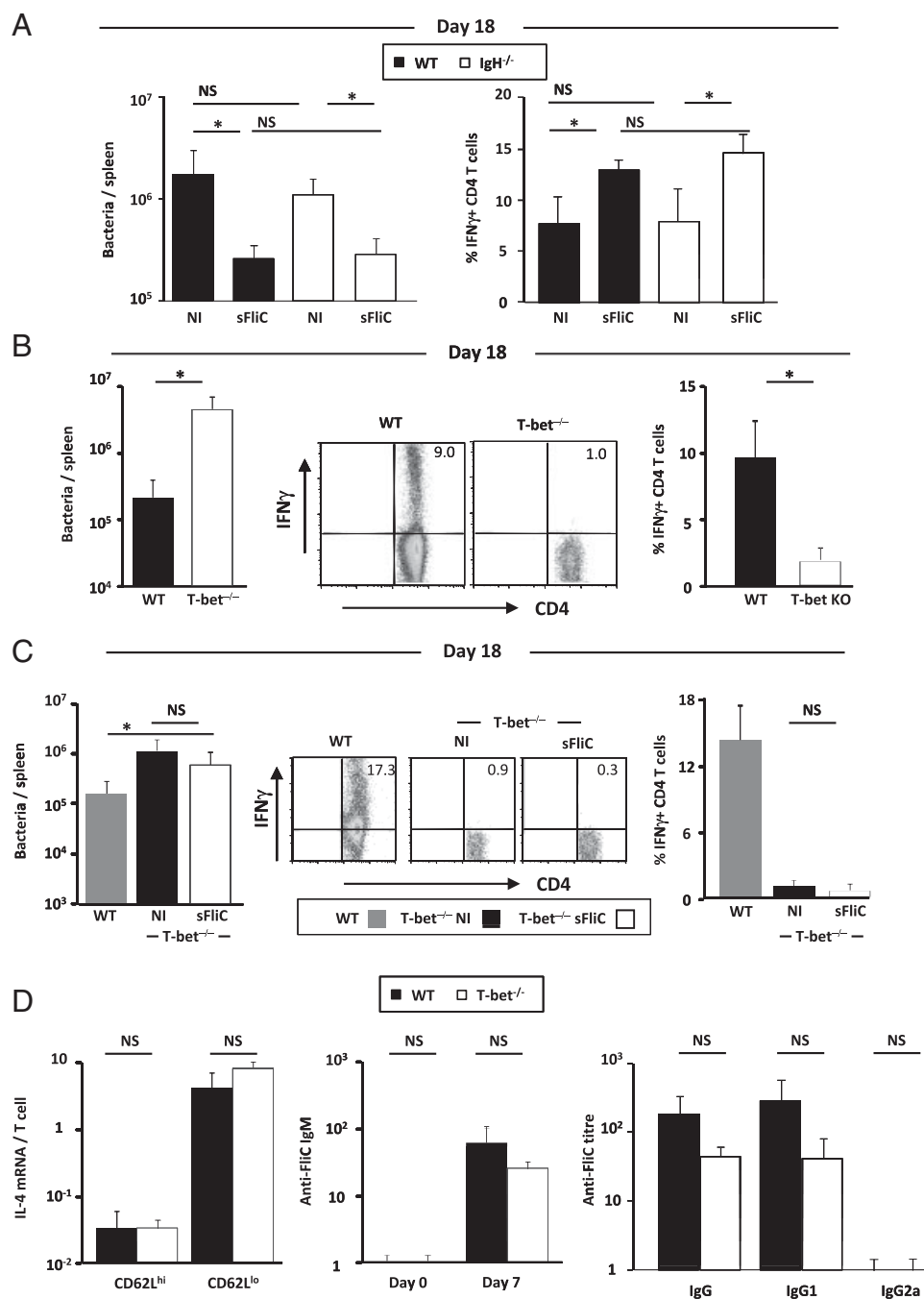


Figure 6. T-bet is required for promoting Th1-mediated clearance after sFliC immunization but not for the induction of Th2 responses. (A) Splenic bacterial numbers (left) and proportions of IFN- γ ⁺ splenic CD4⁺ T cells in NI or sFliC-immunized (20 μ g for 35 days) WT or B-cell-deficient (IgH^{-/-}) mice infected for 18 days i.p. with 5×10^5 STm. (B) WT and T-bet-deficient mice were infected i.p. with 5×10^5 STm for 18 days and splenic bacterial numbers enumerated (left graph) and intracellular IFN- γ production by splenic CD4⁺ T cells assessed, shown as representative FACS panels and right graph. (C) NI WT or NI or sFliC-immunized (20 μ g for 35 days) T-bet-deficient mice were infected i.p. with 5×10^5 STm for 18 days. Splenic bacterial numbers (left) and IFN- γ production by CD4⁺ T cells was assessed by intracellular FACS in CD4⁺ T cells (anti-CD3 stimulation with anti-CD28; centre and right). Groups contained four mice. (D) Left: IL-4 mRNA expression in FACS-sorted splenic WT and T-bet-deficient CD62L^{hi} and CD62L^{lo} CD4⁺ T cells mice 4 days after i.p. immunization with 20 μ g sFliC, CD4 T cells from NI mice had negligible IL-4 mRNA levels (data not shown). Serum anti-FliC IgM 7 days (centre) or IgG and isotypes 35 days (right) from WT and T-bet-deficient mice assessed by ELISA. Graphs show mean and one SD. * $p \leq 0.05$ by the Mann-Whitney test. In all cases, experiments are representative of ≥ 2 repeats.

Th1 regulator T-bet despite sFliC not inducing T-bet (Fig. 2). To confirm that T-bet was required for bacterial clearance at the day 18 time point [12], when sFliC promotes bacterial clearance, we

infected WT and T-bet-deficient mice with 5×10^5 STm and found bacterial numbers were higher in the absence of T-bet and IFN- γ production in CD4⁺ T cells was virtually undetectable

(Fig. 6B). To assess whether T-bet was required for the action of sFliC, NI or T-bet-deficient mice immunized with 20 μ g sFliC for 35 days were infected with 5×10^5 STm for 18 days. Priming with sFliC did not confer any benefit against STm infection in T-bet-deficient mice and IFN- γ production was absent in both sets of mice (Fig. 6C). Thus, the Th2 Ag sFliC enhances STm clearance in a T-bet-mediated manner. Finally, we examined whether this was because T-bet was required for the induction of responses to sFliC. To do this, WT or T-bet-deficient mice were immunized for 4, 7, or 35 days and IL-4 mRNA production assessed in FACS-sorted splenic CD4 $^+$ T cells or serum anti-FliC IgM or IgG assessed by ELISA, respectively. This shows that T-bet was not required for the induction of responses to sFliC (Fig. 6D). Therefore, sFliC induces T-bet-independent Th2 features but requires T-bet to promote STm clearance.

Discussion

By examining immune responses to flagellin, its use as an immunogen and its impact on subsequent infection, we have examined how isolated STm proteins may function as vaccines against STm infection. While sFliC induces potent humoral and cellular immune responses, only cellular immunity was able to, modestly, help control infection. Against expectations, we found that although sFliC induced a primary Th2 response, it could promote Th1-mediated protection that was apparent after the first week of a subsequent STm infection. This suggests that the Th response to sFliC, and by implication other subunit vaccines, can be flexible.

Directing the immune response to an Ag has been a significant focus of research, particularly for adjuvants, because the type of T-cell help initially induced to an Ag can indicate whether the Ag will confer protection. The absolute need for this is challenged by our findings, which suggest that under some circumstances this may not be necessary. The Th2 responses induced to sFliC in vivo develop in the absence of exogenous adjuvants. Few purified proteins have autoadjuvant activity, with proteins such as flagellin, tetanus toxoid, and pertussis toxin having this property. In mice and humans, the responses to these Ag are predominantly Th2 [25, 26, 42, 43] and suggests responses to soluble proteins are likely to be Th2 by default. Nevertheless, the observation that subsequent STm infection resulted in an enhanced Th1 response suggests that T-cell responses show some flexibility. In most instances though, the direction of the Th response may be of secondary importance if protective Ab responses are induced.

The increased numbers of Th1 cells seen after infection of sFliC-primed mice could result from the redirection of Th2 cells to Th1 [44] or derive from primed, yet nondifferentiated T cells [42, 44, 45] or other primed subsets such as IL-17 producing, FoxP3, or BCL6 $^+$ follicular helper T cells [46]. Th2 differentiation to sFliC was the dominant response detected, for there were only low levels of IFN- γ , T-bet, IL-17 ($\leq 0.1\%$ of CD4 $^+$ T cells), BCL6, or FoxP3 $^+$ T cells detected (Fig. 2 and data not shown). It was recently found that LCMV-specific in vitro-primed Th2 cells can redirect to Th1 in vivo and produce IFN- γ in a T-bet-dependent manner [47]. This

important finding shows flexibility in the Th response and the current study augments this by showing that redirection can also occur after immunization. Nevertheless, we suggest that our in vivo findings are also likely to mean that primed, but noncommitted, CD4 $^+$ T cells act as a reservoir from which the enhanced Th1 responses after STm infection of sFliC-immunized mice are derived. This T-cell flexibility after infection of vaccinated animals has been described in other systems [42, 45]. Surprisingly, the Th1 expansion after sFliC immunization required T-bet, despite primary CD4 $^+$ T-cell responses to sFliC being T-bet-independent and T-bet not being required to control other infections [48]. This had suggested to us that the Th1 augmentation after sFliC would be T-bet-independent.

Ab to sFliC was not protective in these studies, in part because STm can phase-switch. Nevertheless, splenic bacterial numbers in sFliC-immunized mice after infection with FliC-locked STm were only modestly reduced, typically <1 log of protection, a level similar to that mediated by CD4 $^+$ T cells at later times. In contrast, effective Ab protection can reduce bacterial numbers by several orders of magnitude [19], and Ab to *Salmonella* is clearly important at preventing infection in humans and mice [16, 19, 49–54]. Immunization with sFliC provided no significant, early, protection against oral infection with STm although there was a trend toward protection seen in the large groups of mice used for these experiments. This suggests that mucosal anti-sFliC responses while probably having some role are not likely to make a substantial impact on controlling early colonization, at least after one immunization with sFliC. The lack of protection by Ab to flagellin in the mouse reflects findings using human sera [54], where antibodies to flagellin had no clear, protective capacity. Thus, structures distal from the cell wall such as flagella may not be efficient targets for Ab-mediated protection. Intriguingly, although binding of flagellin by sFliC-specific sera could inhibit motility, it did not result in shedding or loss of flagella (Fig. 4B and data not shown), suggesting that the bacterium does not necessarily shed flagella when antibody has bound. Thus, Ab to flagellin does not have a significant role in protecting against systemic infection in this model.

Immunity to STm after systemic immunization with flagellin has been described previously [34, 55, 56]. In our experiments, we have focused on how this limited protection is mediated. While this benefit is Ab-independent, CD4 $^+$ T cells are important, but only after the first week of infection. This has two implications. First, since there was no early benefit after sFliC immunization, it suggests that T cells in recall responses do not promote accelerated bacterial clearance after STm infection, a finding we have also seen after porin immunization [19]. A consequence of this is that it suggests that sFliC immunization will confer no significant protection against virulent strains of bacteria since its contribution to immunity is made when infection is well established. Second, it suggests that there is some bioavailability of flagellin throughout infection. While flagellin synthesis, expression, and availability are suppressed during intracellular infection [38, 57, 58], this may not be absolute [59, 60]. Nevertheless, it is unclear whether the modest T-cell-mediated protection after flagellin immunization is because CD4 $^+$ T cells induced after

immunization are not efficient at promoting bacterial killing or whether only a proportion of infected cells contain flagellin-expressing bacteria. Flagellin phase-switching is unlikely to promote immune evasion of T-cell responses since T-cell epitopes are found in conserved regions of *FliC* and *FljB* [32, 34, 38, 36], some of which are found in other serovars such as *Salmonella enteritidis*. This may mean that some T cells after immunization with sFliC will recognize multiple *Salmonella* serovars.

An important question is why the rate of clearance of attenuated bacteria progresses at a protracted rate in the face of potent CD4⁺ T-cell responses, where massive Th1 expansion occurs rapidly after infection [37, 61]? This may mean it is unwise to focus simply on vaccines that evoke CD4⁺ T-cell-mediated immunity in the absence of protective Ab responses. Ab is likely to reduce the numbers of bacteria that colonize, resulting in a lower peak bacterial burden, essentially affording more time for Th1 cells to clear tissue-residing bacteria. Is flagellin simply just a bad choice of Ag for a vaccine? It is not optimal against systemic infection as it fails to induce protective antibody responses, and the benefits from enhanced Th1-cell-mediated immunity are modest. An ideal subunit vaccine should offer both humoral and cellular protection. This may require the generation of subunit vaccines that have more than one component, each contributing protection via humoral or cell-mediated immunity or both. Live STm have this property, but balancing immunogenicity and toxicity is difficult, and attenuated vaccines may not be effective in certain groups such as infants or those with acquired immunodeficiencies. Whether other T-cell Ag (e.g. [62]) are better remains to be seen since their potential to induce protective Ab needs to be carefully evaluated.

Materials and methods

Mice, bacterial strains, and immunogens

Mice were age (6–12 wk) and sex matched. C57BL/6 mice were from HarlanOLAC and SM1 transgenic mice [36] were from Paul Garside with generous permission from Stephen McSorley. OTII transgenic, TCRβδ⁻, Rag1⁻, B-cell⁻, and T-bet-deficient mice [39] were sourced in-house. Animal studies were performed with ethical and Home Office approval.

STm SL3261 is an attenuated strain and OVA-expressing SL3261 has been described previously [63]. *fliC*⁻*fljB*⁺ and *fliC*⁺*fljB*⁻ strains were generated by P22 transduction of the kanamycin resistance gene from SL3201ON and SL3201OFF into SL3261 (SL3201 strains kindly provided by Dr. Alison O'Brien, Uniformed Services University of the Health Sciences; [64]).

FliC and whole flagella were isolated and purified [25] as an immunoprecipitated, his-tagged recombinant protein or from STm by acid hydrolysis [65] and size-exclusion chromatography. OVA was alum precipitated by standard methods [63]. LPS contamination was typically <1 endotoxin unit/300 µg protein (Sigma Endotoxin Kit).

Immunizations, infections, and opsonization of bacteria

Mice received, i.p., 20 µg sFliC or 50 µg alum-precipitated OVA or live bacteria (5×10^5 – 5×10^6 /mouse in PBS from cultures harvested at OD₆₀₀ = 1.2–1.4). Tissue bacterial burdens were determined by direct culturing. Murine infections using opsonized bacteria were performed as described previously [18, 19], using complement-inactivated sera from WT mice immunized twice with sFliC (boosted at 35 days for 14 days). Viability and lack of agglutination was confirmed by plating. For oral infection, STm (10^{10} /mL) were diluted at a ratio of 1:1 with 3% NaHCO₃ and mice immediately infected with 1×10^9 bacteria by oral gavage.

Bacterial motility assay

Bacterial swimming was assessed using 0.3% agar containing complement-inactivated naïve or sFliC+sera (1:300). STm (OD₆₀₀ 1.4) was injected into the agar and swim zone diameters measured after overnight incubation at room temperature.

Flagellin-specific ELISA

ELISA to detect Ab to sFliC or purified flagella was performed as described previously [25]. Plates were coated at 5 µg/mL, then sera, diluted 1:20 in PBS, was added and diluted stepwise. Primary antibodies were detected using alkaline phosphatase-conjugated, goat anti-mouse antibodies (Southern Biotech), and Sigma-Fast *p*-nitrophenylphosphate. Relative reciprocal titres were calculated by measuring the dilution at which the serum reached a defined OD₄₀₅.

FACS analysis, cell sorting, and the generation of chimeras

Splenic single-cell suspensions were prepared and red cells lysed with ammonium chloride buffer. Sometimes, cells were CFSE-labeled by resuspension at 5×10^7 cells/mL in 5 mM CFSE for 5 min. Cells were blocked with anti-CD16:CD32 before staining with one or more of: CD3-FITC, CD62L-phycoerythrin, CD4-allophycocyanin (APC) (all eBioscience) and CD4-PerCP Cy 5.5 and CD69 (biotinylated; BD Biosciences). SM1 cells were identified using CD45.1-phycoerythrin (eBioscience) or β₂-biotin and SA-PerCP Cy 5.5 (BD Biosciences) or CFSE dilution. Samples were acquired on a FACScalibur cytometer and the data were analyzed using FlowJo Software.

Intracellular cytokine staining for IFN-γ and T-bet was performed by ex-vivo restimulation [37]. Briefly, 2.5×10^7 splenocytes/mL stimulated with purified anti-CD3 (precoated overnight at 10 µg/mL) or sFliC (1 µg/mL) in the presence of 1 µg/mL anti-CD28. Cells were incubated at 37°C for 2.5 h, followed by 2.5 h with GolgiStop. Cells were then surface-stained (CD3, CD4,

and CD62L), fixed and permeabilized with Cytofix/Cytoperm Plus and intracellular staining performed using anti-T-bet-PE (Santa Cruz) or anti-IFN- γ -APC or irrelevant APC-labeled isotype control.

Chimeras were generated through i.p. transfer of total splenocytes (10^7) in 200 μ L PBS from SM1 or OTII BoyJ mice into WT or Rag1-deficient recipients. Sometimes, splenocytes were first labeled with CFSE or CD4⁺ T cells sorted ($\geq 98\%$ purity) using a MoFlo cell sorter before 2×10^5 cells were transferred i.p. into Rag1-deficient mice. Chimeras were immunized or infected 24 h post-transfer.

ELISPOT for the detection of FLiC-specific IFN- γ - or IL-4-secreting SFC

ELISPOT assay for IFN- γ was performed using anti-mouse IFN- γ antibody (XMG 1.2) [42] as capture Ab and biotin anti-mouse IFN- γ for detection. ELISPOT for IL-4 was performed with a mouse IL-4 ELISPOT kit (anti-IL4 16-7041-68; eBioscience). After coating with capture Ab, plates were blocked before adding 4×10^5 splenocytes per well. Cells were either restimulated (5 μ g/mL sFLiC or medium) with 1 μ g/mL of anti-CD28, for 48 h at 37°C before incubation for 2 h at room temperature with biotinylated anti-IL4 (BVD6-24G2) or anti-IFN- γ . Streptavidin-peroxidase was added and signal detected using DAB. Spots counted using an AID ELISPOT Reader and software. Counts were expressed as SFC/ 4×10^5 splenocytes.

Quantification of gene expression

RT-PCR was performed on flow cytometry sorted T cells (2×10^5 cells at $\geq 98\%$ purity) subdivided into effector and naïve populations (based on CD62L expression). In mixed SM1-WT chimeras, SM1 cells were identified based on CFSE or V β 2 staining. RNA was purified using the RNeasy Mini Kit (Qiagen) and reverse transcribed using Superscript III. Real-time PCR for relative gene expression was performed as described previously [25, 63, 66] using 2 \times PCR Master Mix (Applied Biosystems) and the results presented as the relative signal per cell compared with β -actin.

Statistical analysis

Statistical analysis was conducted using the Mann–Whitney nonparametric sum of ranks test using the Analyze-It programme and significance was accepted where $p \leq 0.05$.

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Abbreviations: APC: allophycocyanin · NI: nonimmunized · SFC: spot-forming cells · sFliC: soluble, recombinant FliC protein flagellin · ST: *Salmonella enterica* serovar Typhi · STm: *Salmonella enterica* serovar Typhimurium

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